# Design, Synthesis, and Pharmacological Evaluation of Fluorescent and Biotinylated Antagonists of ρ_1 GABA$_C$ Receptors

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Ⓢ *Supporting Information*

ABSTRACT: The ρ_1 GABA$_C$ receptor is a ligand-gated chloride ion channel that shows promise as a therapeutic target for myopia, sleep disorders, memory and learning facilitation, and anxiety-related disorders. As such, there is a need for molecular probes to understand the role GABA$_C$ receptors play in physiological and pathological processes. To date, no labeled (either radioactive or fluorescent) GABA$_C$ selective ligand has been developed that can act as a marker for GABA$_C$ receptor visualization and localization studies. Herein, we report a series of fluorescent ligands containing different-sized linkers and fluorophores based around (S)-4-ACPBPA [(4-aminocyclopenten-1-yl)-butylphosphinic acid], a selective GABA$_C$ antagonist. One of these conjugates, (S)-4-ACPBPA-C5-BODIPY (**13**), displayed moderate potency (IC$_{50}$ = 58.61 μM) and selectivity (>100 times) for ρ_1 over $\alpha_1\beta_2\gamma_{2L}$ GABA$_A$ receptors. These conjugates are novel lead agents for the development of more potent and selective fluorescent probes for studying the localization and function of GABA$_C$ receptors in living cells.

KEYWORDS: *Human ρ_1 GABA$_C$ receptors, CNS-related disorders, GABA$_C$ antagonists, fluorescent and biotinylated probes, homology modeling and docking*
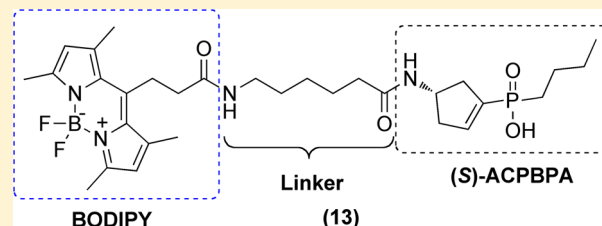
γ-Aminobutyric acid **1** [GABA (Figure 1)] is the most abundant inhibitory neurotransmitter in the mammalian central nervous system (CNS) and is essential for the overall balance between neuronal excitation and inhibition.[1,2] GABA released from GABAergic axon terminals influences neurons via GABA$_A$, GABA$_B$, and GABA$_C$ (GABAρ) receptors. These receptors are grouped on the basis of their subunit composition, gating properties, and pharmacological profiles. GABA$_A$ and GABA$_C$ receptors are ligand-gated chloride ion channels that mediate fast synaptic inhibition when activated by GABA,[1] whereas GABA$_B$ receptors are G-protein-coupled receptors that mediate slow, longer-lasting synaptic inhibition by increasing potassium and decreasing calcium conductances when activated by GABA.[3]

Ionotropic GABA$_A$ receptors are transmembrane protein complexes composed of five heteropentameric subunits. To date, 16 human GABA$_A$ receptor subunits have been identified and classified as α (α_1–α_6), β (β_1–β_3), γ (γ_1–γ_3), δ, ε, π, and θ.[4] In contrast, ionotropic GABA$_C$ receptors have pharmacology, physiology, and subunit compositions distinct from those of GABA$_A$.[5,6] In mammals, GABA$_C$ receptors are composed of ρ subunits (ρ_1–ρ_3), forming homopentameric assemblies of five ρ subunits (ρ_1, ρ_2, or ρ_3 subunits) or pseudoheteropentameric complexes comprising different ρ subunits (a combination of ρ_1 and ρ_2 or ρ_2 and ρ_3 subunits).[5,7,8] GABA$_C$ receptors are insensitive to bicuculline and (−)-baclofen, selectively activated by (+)-CAMP **3** [(+)-cis-2-(aminomethyl)-cyclopropanecarboxylic acid (Figure 1)] and 5-Me-I-4AA **4** (5-methyl-1H-imidazole-4-acetic acid), and inhibited by TPMPA **5** (1,2,5,6-tetrahydropyridine-4-yl-methyl-phosphinic acid), (S)-4-ACPBPA **6** {[(S)-4-aminocyclopenten-1-yl]-butyl-phosphinic acid}[9] and 3-GOHP **8** [3-(guanido)-1-oxo-1-hydroxy-phospholane].[10] This clearly indicates that the GABA binding sites of GABA$_C$ and GABA$_A$ receptors are not identical.

GABA$_A$ receptors are widely distributed in the CNS,[1] whereas GABA$_C$ receptors are mainly expressed in the superior colliculus,[11] cerebellum,[12] lateral amyglada,[13] hippocampus (strong ρ_2 subunit expression),[14] and, most prominently, the retina (strong ρ_1 subunit expression).[7] Various studies show that GABA$_C$ receptors are potential therapeutic targets for myopia, sleep disorders, managing memory-related disorders, and peripheral antinociception.[5,13] However, understanding the role of GABA$_C$ receptors and information about the processes triggered by ligand–receptor interactions in living cells are still limited because of the lack of pharmacological tools.

Fluorescent ligands have proven to be useful tools offering a wealth of information about the mapping or identification of ligand binding sites,[15,16] the mechanism of ligand binding,[17] the physical nature of the binding pocket, the movement and
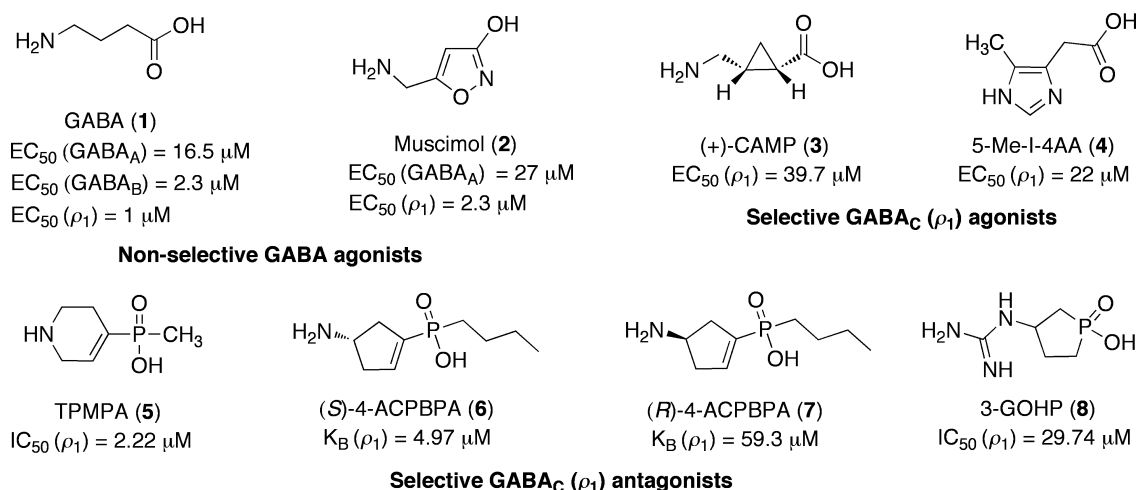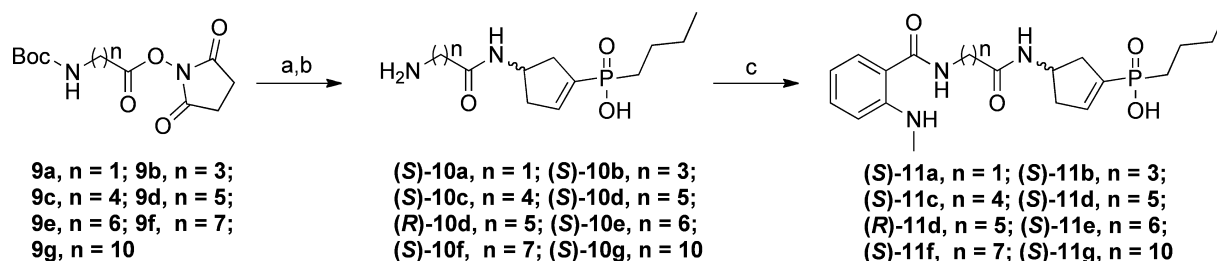
Figure 1. Structures of GABA (**1**), the GABA$_A$ agonist and GABA$_C$ partial agonist muscimol (**2**), the GABA$_C$ selective agonists (+)-CAMP (**3**) and 5-Me-I-4AA (**4**), and GABA$_C$ selective antagonists TPMPA (**5**), (*S*)-4-ACPBPA (**6**), (*R*)-4-ACPBPA (**7**), and 3-GOHP (**8**).

Scheme 1. Synthesis of Fluorescent ρ_1 GABA$_C$ Antagonists (*S*)-11a–g and (*R*)-11d[a]

[a]Reagents and conditions: (a) (*S*)-4-ACPBPA **6** or (*R*)-4-ACPBPA **7** [for (*R*)-**10d**], NaHCO$_3$, water/DME/THF (1:1:0.3), room temperature for 16 h, 81−89%; (b) TFA/DCM (1:1), room temperature for 1−2 h, 92−98%; (c) NMA *N*-succinimidyl ester, NaHCO$_3$, water/DME/THF (1:1:0.3), room temperature for 16 h, 75−84%.

internalization of receptors in living cells,[18] and the localization as well as visualization of the labeled receptors.[19] Biotinylated probes are useful for the isolation and purification of target proteins of the receptor fragment for crystallization and X-ray studies of the particular receptor-binding site,[20] the localization and visualization of the receptor by using antibodies,[21] and the study of ligand–protein interactions in living cells.[22]

In addition, fluorescent and biotinylated probes represent a faster, safer, and less expensive alternative to radioligands in receptor studies, circumventing several drawbacks associated with radioligand studies such as health and safety concerns and the need for a large number of cells with strong receptor expression.[23] Thus, the identification of fluorescent or biotinylated ligands for GABA$_C$ receptors is of great interest. The development of these tagged ligands usually starts with the selection of a potent and selective pharmacophore, which is then conjugated with the desired tag through an appropriate linker.
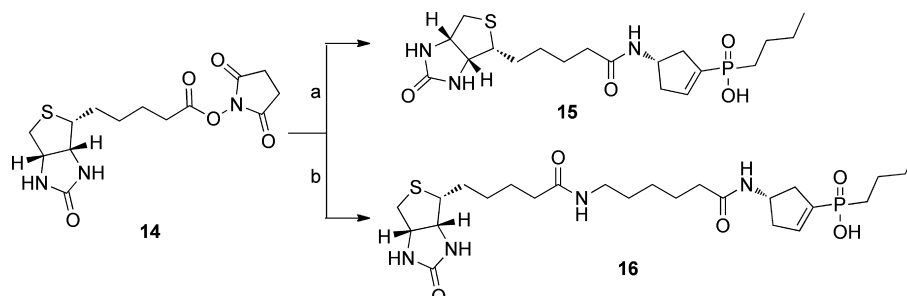
To date, only a few fluorescent and biotinylated GABA$_B$ receptor antagonist ligands have been reported,[24] and N-acylation of the amino moiety of muscimol (**2**), a widely used GABA$_A$ agonist that it is also a potent partial agonist at human ρ_1 GABA$_C$ receptors with fluorophores, has been shown to result in ligands that bind to the GABA$_A$ receptors.[25] Vu et al. presented evidence that muscimol linked through a 6-aminohexanoyl chain to biotin (muscimol-biotin; EC$_{50}$ = 20 μM) and BODIPY (muscimol-BODIPY) retained substantial agonist activity compared to muscimol (**2**; EC$_{50}$ = 2 μM) at

GABA$_C$ receptors,[26] indicating a steric tolerance in the region around the ligand's amine binding site of the GABA$_C$ receptor. To the best of our knowledge, no selective fluorescent ligand for GABA$_C$ receptors has been described in the literature. Therefore, we envisioned that incorporating fluorophores or the versatile biotin moiety into selective GABA$_C$ ligands could lead to selective probes. Herein, we report the design, synthesis, and pharmacological evaluation of the selective fluorescent and biotinylated probes for ρ_1 GABA$_C$ receptors.

We selected two of our previously reported selective and potent GABA$_C$ antagonists, (*S*)-4-ACPBPA **6** and (*R*)-4-ACPBPA **7** (Figure 1), for the design of the fluorescent GABA$_C$ antagonists.[9] Both were synthesized according to our previously published protocol.[9] The length of the spacer chain is known to significantly impact the affinity of ligand–fluorophore conjugates for the receptors; therefore, we chose a linker size that varied from 0 to 10 carbons to introduce molecular flexibility into the conjugated fluorescent ligand. We incorporated fluorescent groups with relatively small to large molecular volumes to study the effect of perturbation on receptor affinity. We used the fluorophores NMA (*N*-methylanthranilic acid), BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene), and NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yl) chloride, which are known to have different excitation wavelengths and distinctive properties, to diversify fluorescence experiments. BODIPY has been extensively utilized for the development of fluorescent probes because of its distinctive and useful features, including hydrophobicity, photochemical

Scheme 2. Synthesis of Fluorescent ρ_1 GABA$_C$ Antagonists 12 and 13[a]
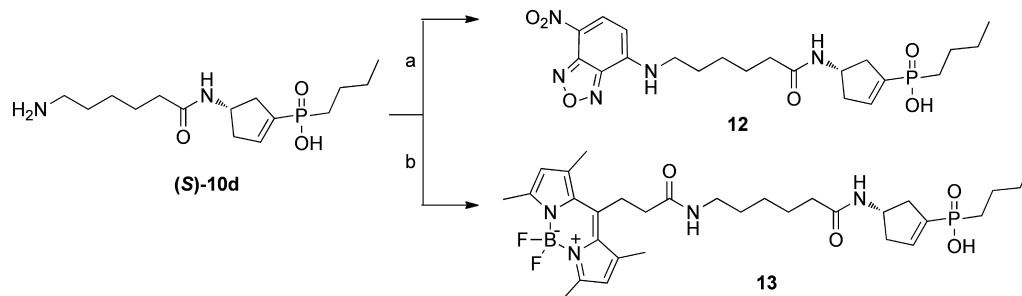
[a]Reagents and conditions: (a) 7-nitrobenz-2-oxa-1,3-diazol-4-yl chloride (NBD-Cl), K$_2$CO$_3$, DMF, room temperature for 24 h, 76%; (b) BODIPY N-succinimidyl ester, DIPEA, DMF, room temperature for 24 h, 72%.

Scheme 3. Synthesis of Biotinylated ρ_1 GABA$_C$ Antagonists 15 and 16[a]

[a]Reagents and conditions: (a) (S)-4-ACPBPA 6, DIPEA, DMF, room temperature for 24 h, 75%; (b) (S)-10d, DIPEA, DMF, room temperature for 24 h, 81%.

Table 1. Pharmacological Evaluation of Fluorescent and Biotinylated Probes[a]

compound	spacer	human ρ_1 GABA$_C$ receptor % inhibition and IC$_{50}$ μM (95% confidence interval)		human $\alpha_1\beta_2\gamma_{2L}$ GABA$_A$ receptor % inhibition at 300 μM
		300 μM	600 μM[d]	
(S)-4-ACPBPA (6)		97 ± 3%[b]		inactive[g]
		IC$_{50}$ = 9.76, K_B = 4.97 μM[c]		
(R)-4-ACPBPA (7)		92 ± 7%[b]		inactive[g]
		K_B = 59.3 μM[c]		
(S)-4-ACPBPA-C1-NMA [(S)-11a]	1		8 ± 2%	
(S)-4-ACPBPA-C3-NMA [(S)-11b]	3		23 ± 4%	
(S)-4-ACPBPA-C4-NMA [(S)-11c]	4		31 ± 2%	
(S)-4-ACPBPA-C5-NMA [(S)-11d]	5		62 ± 7%	inactive[g]
(R)-4-ACPBPA-C5-NMA [(R)-11d]	5		56 ± 9%	inactive[g]
(S)-4-ACPBPA-C6-NMA [(S)-11e]	6		55 ± 3%	
(S)-4-ACPBPA-C7-NMA [(S)-11f]	7		24 ± 8%	
(S)-4-ACPBPA-C10-NMA [(S)-11g]	10		8 ± 3%	
(S)-4-ACPBPA-C5-NBD (12)	5	IC$_{50}$ = 281.42 (274.96−332.11)[e]		inactive[g]
		IC$_{50}$ = 193.27 (181.67−224.81)[f]		
(S)-4-ACPBPA-C5-BODIPY (13)	5	IC$_{50}$ = 103.14 (95.36−114.87)[e]		inactive[g]
		IC$_{50}$ = 58.61 (45.37−71.63)[f]		
(S)-4-ACPBPA-C0-biotin (15)	0	inactive[b]		
(S)-4-ACPBPA-C5-biotin (16)	5	IC$_{50}$ = 147.92 (139.45−174.13)[e]		9 ± 7%[g]
		IC$_{50}$ = 76.54 (68.33−102.96)[f]		

[a]Determined electrophysiologically in *Xenopus laevis* oocytes expressing the human ρ_1 GABA$_C$ receptor or human $\alpha_1\beta_2\gamma_{2L}$ GABA$_A$ receptors as previously described.[9,10] [b]Activity or percent inhibition by 300 μM compound of the current produced by a submaximal concentration of GABA (1 μM, EC$_{50}$). [c]Data from ref 9. [d]Percent inhibition by 600 μM compound of the current produced by a submaximal concentration of GABA (1 μM, EC$_{50}$), with a 5 min preincubation. [e]IC$_{50}$ values for compounds without preincubation. [f]IC$_{50}$ values for compounds with a 5 min preincubation. [g]Activity or percent inhibition by 300 μM compound of the current produced by a submaximal concentration of GABA (30 μM, EC$_{50}$) without and with a 5 min preincubation. All data are means ± the standard error of the mean (n = 3 oocytes).

stability, insensitivity to changes in experimental conditions, efficient uptake into cell membranes, and high extinction coefficient and fluorescence quantum yield.[27] Initially, we synthesized fluorescent probes using the potent and selective
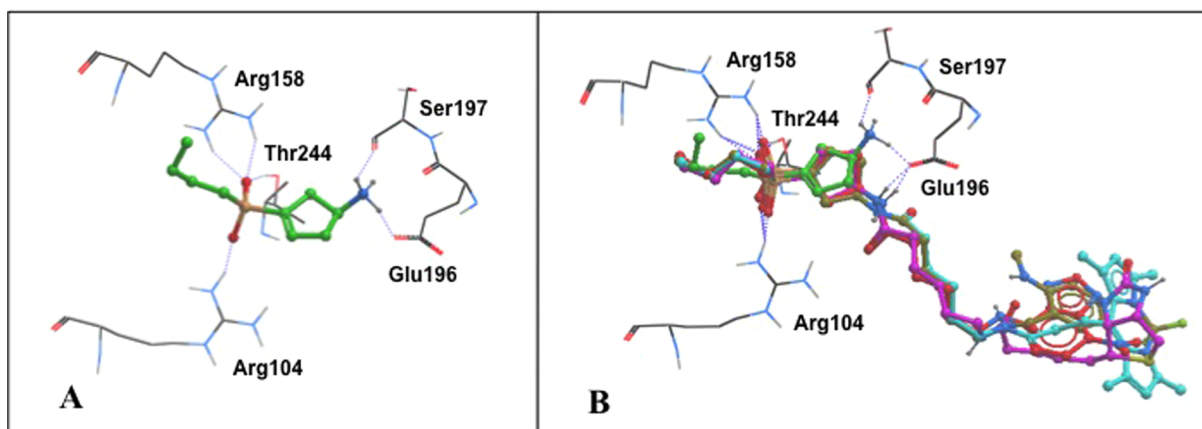
Figure 2. View of the ρ_1 GABA$_C$ ligand-binding site with predicted binding modes. (A) Close-up of (S)-4-ACPBPA **6** (green carbons). (B) Close-up of (S)-4-ACPBPA-C5-NMA (S)-**11d** (brown carbons), (S)-4-ACPBPA-C5-NBD **12** (red carbons), (S)-4-ACPBPA-C5-BODIPY **13** (aqua blue carbons), and (S)-4-ACPBPA-C5-biotin **16** (magenta carbons) that are overlaid on (S)-4-ACPBPA **6** in the GABA$_C$ binding site, with the linker and fluorophores external to the orthosteric site.

GABA$_C$ antagonist (S)-4-ACPBPA **6** by linking the small fluorophore NMA through linkers of various sizes.

The synthesis of (S)-4-ACPBPA-NMA- and (R)-4-ACPBPA-C5-NMA-conjugated target compounds (S)-**11a–g** and (R)-**11d** is depicted in Scheme 1. N-Succinimidyl esters **9a–g** were prepared by protection of the amine function of the amino acids with a *tert*-butyloxycarbonyl group (Boc), followed by activation of the acid function of the amino acids with an N-hydroxysuccinimide (NHS) (see the Supporting Information for the experimental details). The activated N-succinimidyl esters **9a–g** were further reacted with the GABA$_C$ antagonist (S)-4-ACPBPA **6** in an aqueous NaHCO$_3$ solution at room temperature, and subsequent removal of the Boc group using TFA in CH$_2$Cl$_2$ to afford compounds **10a–g** in moderate to good yields. Target compounds (S)-**11a–g** were obtained by amide coupling of compounds (S)-**10a–g** with NHS-activated fluorophore NMA (NMA N-succinimidyl ester) in an aqueous NaHCO$_3$ solution at room temperature. (R)-**10d** was obtained from **9d** as described above for the preparation of (S)-**10d**. (R)-**11d** was synthesized by reaction of (R)-4-ACPBPA **7** with the previously prepared ester (R)-**10d** under basic conditions at room temperature.

Fluorescent compounds **12** and **13** containing the NBD and BODIPY moieties were prepared as depicted in Scheme 2. Condensation of 7-nitrobenz-2-oxa-1,3-diazol-4-yl chloride (NBD chloride) with (S)-**10d** in a K$_2$CO$_3$/DMF mixture at room temperature afforded target compound **12** in moderate yield. The synthesis of BODIPY N-succinimidyl ester (see the Supporting Information for the experimental details) was achieved in two steps according to a previously described method.[28] Compound **13** was obtained in moderate yield by reaction of BODIPY succinimidyl ester and the primary amine of (S)-**10d** in a DIPEA/DMF mixture at room temperature.

Biotinylated conjugates **15** and **16** were prepared according to Scheme 3. Formation of an amide bond between biotin N-succinimidyl ester **14** and the corresponding primary amine of (S)-4-ACPBPA **6** and compound (S)-**10d** in a DIPEA/DMF mixture at room temperature afforded biotinylated conjugates **15** and **16**, respectively.

We examined the functional characterization of fluorescent and biotinylated probes on human recombinant GABA receptors expressed in *Xenopus* oocytes using the two-electrode voltage clamp method (Table 1).[9,10] All 12 probes [(S)-**11a–g**,

(R)-**11d**, **12**, **13**, **15**, and **16**] were evaluated for activity alone and in the presence of GABA on GABA$_A$ ($\alpha_1\beta_2\gamma_{2L}$), and ρ_1 GABA$_C$ receptors to determine whether they behave as agonists, antagonists, or modulators.

During the initial pharmacological screening, we found that stable and reproducible levels of inhibition of GABA (1 μM) at ρ_1 GABA$_C$ receptors were reached only after a 5 min preincubation with compounds (S)-**11a–g** and (R)-**11d** (see the Supporting Information for details). The preincubation needed for some of the compounds for reaching stable and reproducible levels of inhibition of GABA may be due to a molecule's size and lipophilicity needing additional time to diffuse into the binding site. In the (S)-4-ACPBPA-NMA series where the NMA group was attached to the primary amine of the ligand through an aminoalkanoyl chain, we observed that compounds with a chain length of fewer than five carbons [compounds (S)-**11a–c**] exhibited weak antagonist activity, compared to compound (S)-**11d** with a five-carbon chain. The activity for compounds (S)-**11e–g** decreased with an increasing chain length. These compounds had a chain length of 6–10 carbons, indicating that a linker of five carbons is optimal. The decrease in activity for compounds (S)-**11a** and (S)-**11b** (spacer size of fewer than four carbons) could be explained by the relative rigidity caused by the amido-containing chain with the ligand moiety at the binding site. With spacer sizes of more than six carbons, the fluorescent tag may occupy a less favorable area, resulting in a decrease in inhibitory activity.

The optimal chain length of the linker is important for the introduction of molecular flexibility for more convenient positioning of the ligand into the receptor. After optimization of the chain length for fluorescent probes, we linked (R)-4-ACPBPA through a 6-aminohexanoyl chain to NMA [(R)-**11d**] and evaluated its activity on ρ_1 GABA$_C$ receptors. (R)-4-ACPBPA **7** is 12-fold less potent than (S)-4-ACPBPA **6** at the ρ_1 GABA$_C$ receptor (Figure 1).[9] However, (R)-4-ACPBPA-C5-NMA (R)-**11d** exhibited activity almost equal to that of (S)-4-ACPBPA-C5-NMA (S)-**11d**, indicating N-acylation of the amine removed the differential activity of enantiomers.

We also evaluated NBD and BODIPY (**12** and **13**, respectively) containing probes on both ρ_1 GABA$_C$ and $\alpha_1\beta_2\gamma_{2L}$ GABA$_A$ receptors with and without preincubation (Table 1). (S)-4-ACPBPA-C5-NBD **12** exhibited poor potency (IC$_{50}$ = 281.42 μM) when tested without preincubation, but its

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activity was slightly improved (IC$_{50}$ = 193.27 μM) with a 5 min preincubation at ρ_1 GABA$_C$ receptors. (S)-4-ACPBPA-C5-BODIPY **13** displayed moderate and improved potency (IC$_{50}$ = 58.61 μM with preincubation, and IC$_{50}$ = 103.14 μM without preincubation) at ρ_1 GABA$_C$ receptors. The increase in activity for (S)-4-ACPBPA-C5-BODIPY **13** but not for (S)-4-ACPBPA-C5-NMA (S)-**11d** and (S)-4-ACPBPA-C5-NBD **12** indicates that bulkier fluorophores linked by a 6-aminohexanoyl chain are more favorable for activity. These results confirmed the prediction of molecular modeling studies that the large hydrophobic region of the receptor is located adjacent to the binding site of the basic amino group of (S)-4-ACPBPA. As expected, probes (S)-**11d**, (R)-**11d**, **12**, and **13** were found to be inactive (at 300 μM) at $\alpha_1\beta_2\gamma_{2L}$ GABA$_A$ receptors.

We also evaluated biotinylated probes of (S)-4-ACPBPA (compounds **15** and **16**) on the ρ_1 GABA$_C$ receptor. Biotin-coupled probes are suitable for subcellular localization of receptors because biotin forms a tight complex with strept(avidin),[21] which can be visualized by confocal microscopy using antibodies. Direct attachment of the biotin group to (S)-4-ACPBPA was deleterious for activity, (compound **15** was inactive at 300 μM). However, a linker length of five carbons (compound **16**) is sufficient to restore moderate activity (IC$_{50}$ = 76.54 μM with preincubation, and IC$_{50}$ = 147.92 μM without preincubation) at ρ_1 GABA$_C$ receptors. These findings represent the first demonstration of electrophysiological activity by N-acyl derivatives of selective antagonists (S)-4-ACPBPA **6** and (R)-4-ACPBPA **7**.

To identify structural determinants for the observed activity for the ρ_1 GABA$_C$ receptor, we flexibly docked the structures of (S)-4-ACPBPA **6** and fluorescent and biotinylated probes (S)-4-ACPBPA-C5-NMA (S)-**11d**, (S)-4-ACPBPA-C5-NBD **12**, (S)-4-ACPBPA-C5-BODIPY **13**, and (S)-4-ACPBPA-C5-biotin **16** into the ligand-binding site of a ρ_1 GABA$_C$ homology model.[29] Figure 2B shows predicted binding modes for (S)-4-ACPBPA-C5-NMA (S)-**11d**, (S)-4-ACPBPA-C5-NBD **12**, (S)-4-ACPBPA-C5-BODIPY **13**, and (S)-4-ACPBPA-C5-biotin **16**, which show modes of binding for probe-linked (S)-4-ACPBPA similar to that of parent (S)-4-ACPBPA **6** (Figure 2A). The binding affinity is largely ascribed to various electrostatic interactions,[29−31] including (i) a salt bridge interaction between the phosphinic acid and Arg104 and Arg158,[29,30] (ii) hydrogen bond contacts of the amine of parent ligand or amide of probes with Glu196,[9] and (iii) hydrogen bond contacts with Thr244.[31] The docked model of compounds **6**, (S)-**11d**, **12**, **13**, and **16** (Figure 3) orients the (S)-4-ACPBPA moiety in the active site cavity of the ρ_1 GABA$_C$ receptor (shown with wire mesh) and a flexible spacer between the (S)-4-ACPBPA core and probe where the probe is pointing out of or residing in the noninteractive binding site of the receptors, which cannot be ruled out at this stage. The fluorophores or biotin tags are shown by molecular modeling studies to reside in a pocket away from the orthosteric site or extend into the extracellular space.

In conclusion, we have designed and synthesized for the first time fluorescent and biotinylated probes of selective antagonists for ρ_1 GABA$_C$ receptors. Pharmacological studies show that **12**, **13**, and **16** exhibit activity similar to that of the previously reported muscimol-based probes,[25,26] with the added advantage that they are selective GABA$_C$ receptors. These results offer new knowledge regarding the binding site and receptor flexibility of GABA$_C$ receptors. The described fluorescent and biotinylated probes will be useful tools for localizing,

Figure 3. View of the (S)-4-ACPBPA moiety (wire mesh) in the active site cavity of the ρ_1 GABA$_C$ receptor model and flexible spacer between the (S)-4-ACPBPA core and probes pointing away from the orthosteric site.

visualizing, and studying the physiopathological processes of GABA$_C$ receptors. Further optimization of probes at amino and phosphinic acid moieties of (S)-4-ACPBPA, in vitro pharmacological evaluation, and fluorescent spectroscopic characterizations of all the probes are underway.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthetic method, characterization of compounds, pharmacology, and molecular modeling. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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